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Cancer Mediated Osteolysis

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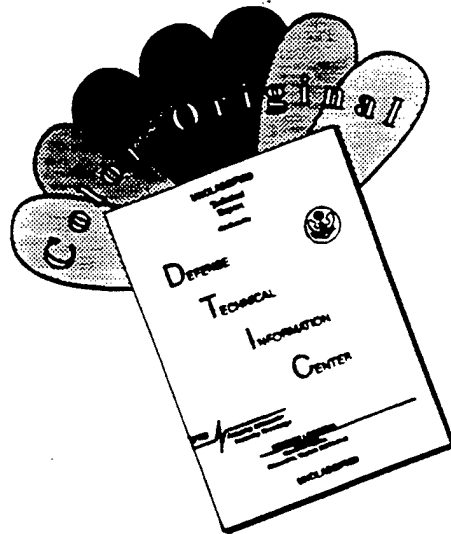
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Therese A. Mune 10/28/96
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INTRODUCTION

NATURE OF THE PROBLEM AND BACKGROUND OF PREVIOUS WORK

Breast Cancer, Hypercalcemia and Osteolysis

Breast cancer is associated with significant morbidity in the skeleton. Specifically, breast cancer can involve bone through both metastatic and humoral mechanisms. Metastases to bone are more commonly osteolytic than osteoblastic and are responsible for the complications of bone pain, pathologic fracture, hypercalcemia and nerve compression syndromes that many breast cancer patients suffer from (1). Eighty-four per cent of patients dying of breast cancer have bone metastases (2).

Hypercalcemia is commonly associated with breast cancer, occurring in up to 40% of afflicted women during the course of their disease (2,3). Skeletal destruction by metastatic tumor has been felt to be the major mechanism responsible for hypercalcemia (3). Increased osteoclastic bone resorption in areas surrounding breast cancer metastasis has been documented histologically (4,5) suggesting that factors secreted by breast cancer cells can locally activate osteoclasts. Recent evidence, however, suggests that osteolytic bone metastasis may not be the only mechanism responsible for breast cancer hypercalcemia and that humoral mechanisms may contribute in as much as 30-60% of the cases (6-8). In one study, 15% of 147 hypercalcemic breast cancer patients had no bone metastases (9).

PTHrP and Breast Cancer

Parathyroid hormone-related protein (PTHrP) is a major mediator of humoral hypercalcemia of malignancy, due to its PTH-like actions. This protein was purified in 1987 from human lung cancer (10), breast cancer (11) and renal cell carcinoma (12) simultaneously by several independent groups. Cloning and expression followed shortly thereafter (13).

PTHrP has since been extensively studied and found to have many similarities to PTH. It has 70% homology to the first 13 amino acids of the N-terminal portion of PTH (13), binds to PTH receptors (14) and shares similar biologic activity to PTH (15). Specifically, it stimulates adenylate cyclase in renal and bone systems (11,12,15-17), increases renal tubular reabsorption of calcium and osteoclastic bone resorption (16,17), decreases renal phosphate uptake (15,16,18) and stimulates 1α -hydroxylase (15). PTHrP has been found in a variety of tumor types as well as normal tissue (19-22). The widespread expression of PTHrP in normal as well as malignant tissue was the first evidence that the hormone has a role in normal physiology. In addition to the PTH-like effects, PTHrP has many non-PTH-like properties (23), some of which include regulation of placental calcium transport (22), possible establishment of bone metastasis in breast cancer (24,25), and autocrine regulation of the growth of some tumors (26). The regulation of PTHrP is poorly understood, but factors such as prolactin (27), glucocorticoids, $1,25(\text{OH})_2\text{D}_3$ (28), epidermal growth factor (28), $\text{TGF}\alpha$ (29), $\text{TGF}\beta$ (30), estrogen (31-34) and stretch (35) have been shown to regulate gene expression and extracellular calcium concentration has been shown to control the production of PTHrP in vitro in Leydig tumor cells (36).

It is now clear that PTHrP is a significant factor in mediating hypercalcemia in breast cancer (37). One of the 3 tumors from which PTHrP was originally purified was a breast cancer from a patient with humoral hypercalcemia of malignancy (11). PTHrP was detected by immunohistochemical staining in 60% of 102 invasive breast tumors removed from normocalcemic women, but not in normal breast tissue (24). By immunohistochemistry (25) and in situ hybridization (38), it was detected in 12 of 13 breast cancer metastases in bone prompting speculation that production of PTHrP as a bone-resorbing agent may contribute to the ability of breast cancers to grow as bone metastasis. Along these lines, Bundred and colleagues found positive immunohistochemical staining for PTHrP in 56% of 155 primary breast tumors from normocalcemic women and PTHrP positivity was related to the development of bone metastases (39). Additionally, 65-92% of hypercalcemic breast cancer patients (with and without bone metastasis) had detectable plasma PTHrP concentrations by radioimmunoassay (RIA) similar to those documented in patients with humoral hypercalcemia of malignancy due to non-breast tumors (40,41).

PTHrP in Nonmalignant Breast Disease

In addition to its role in malignancy, PTHrP is important in the normal physiology of breast (42). It is expressed in lactating mammary tissue (43) and secreted into milk at concentrations 10,000-100,000 times greater than plasma concentrations of humans with malignancy-associated hypercalcemia (44-48). Suckling increases PTHrP gene expression and this appears to be mediated through prolactin (49). Estrogen has been shown to increase PTHrP expression in uterine tissue and *in vitro* studies suggest that there may be estrogen response elements present in the PTHrP gene (50-53). Increased plasma PTHrP concentrations have been described in at least 2 patients with the rare syndrome of lactational hypercalcemia (54-56). Animal studies have demonstrated a PTHrP gradient across the mammary gland in lactating goats (48) indicating that PTHrP may gain access to the maternal circulation during lactation. In support of this, a recent clinical study has shown detectable plasma PTHrP concentrations in 63% of breast-feeding mothers while similar measurements in bottle-feeding control mothers were undetectable (57). Thus, PTHrP may be responsible for mobilizing calcium from maternal bone for use in milk production and it may be the implicating factor in lactation-associated bone loss (58).

PTHrP as a Growth Regulator

PTHrP is produced in relatively low concentrations in breast myoepithelial cells (59). A transgenic mouse model, in which PTHrP is over expressed in skin and breast myoepithelial cells through the use of a human keratin promoter, has demonstrated breast hypoplasia. Specifically, female transgenics had a severe reduction in the number of albeit normal terminal ducts and acini in the breast suggesting that PTHrP may play a role in regulating ductular proliferation and/or differentiation during mammaryogenesis (60). These mice also had failure of normal hair follicle development indicating a similar role for PTHrP in the skin.

Along those lines, disruption of PTHrP expression in a normal keratinocyte cell line, using antisense technology, results in enhanced growth of the cells in culture (61). *In vivo*, homozygous mice for the PTHrP null mutation are born with a multitude of skeletal abnormalities, including defects in the bone growth plate (62). These findings, along with those of the above described transgenic mice, suggest that either over- or under- expression of PTHrP in normal cells result in abnormalities of growth and possibly differentiation.

In malignant cells, PTHrP has been shown to act as an autocrine growth factor in a renal cell carcinoma cell line (26) and more recently, in a squamous cell carcinoma line (63). There are no reported studies on the role of PTHrP as an autocrine growth factor in breast cancer.

Regulation of PTHrP by Other Tumor-associated and Bone-derived Growth Factors

Other tumor-associated growth factors as well as bone-derived growth factors may be important regulators of PTHrP expression in both malignant and non-malignant tissue. Epidermal growth factor has been shown to increase PTHrP expression in a keratinocyte cell (64) line while TGF- α , a breast cancer tumor product (65), enhances PTHrP expression in a human squamous cell carcinoma of the lung (29). Moreover, other tumor-associated factors may modulate the end organ effects of PTHrP. TGF- α enhances the hypercalcemic effects of PTHrP in an animal model of malignancy-associated hypercalcemia (66) and it can modulate the renal and bone effects of PTHrP as well (67,68). Additionally, TGF- β , which is present in high concentrations in the bone microenvironment, has been shown to enhance secretion of and stabilize the message for PTHrP in a renal cell carcinoma (30) as well as in an epidermal squamous cell carcinoma (69).

Implications of PTHrP Status in Breast Cancer

These findings have important implications for the ability of breast cancer to affect the skeleton. First, breast cancers expressing PTHrP in addition to other tumor-associated factors, such as TGF- α (65), may be more likely to affect the skeleton through humoral and osteolytic mechanisms if the co-expressed factor enhances PTHrP expression in the primary tumor. Second, if estrogen regulates PTHrP expression in breast cancer cells as it does in other tissues, estrogen receptor positive tumors may preferentially express PTHrP. Finally, growth of breast cancer cells in bone may be enhanced if the tumor cells express PTHrP. TGF- β , as well as other bone derived growth factors, are present in high concentration in the bone microenvironment (70) and are released from bone during the process of osteoclastic bone resorption (71). PTHrP expression in breast cancer cells lodged in bone is likely to be increased in the presence of TGF- β . In this scenario, osteoclastic bone resorption is increased further causing release of more TGF- β and other growth factors into the bone microenvironment leading to further enhancement of PTHrP expression in the breast cancer cells. If PTHrP acts as an autocrine growth factor in breast cancer cells, as it does in some tumor models, then tumor growth would be enhanced as well. The clinical findings of an increased incidence of PTHrP expression in bone compared with other sites by Powell and colleagues (25,38) supports the notion that production of PTHrP as a bone resorbing agent may contribute to the ability of breast cancers to grow as bone metastases.

If PTHrP expression in the primary breast tumor indicates a propensity to metastasize to bone due to its potent bone resorbing capability, early treatment with inhibitors of bone resorption is likely to prevent or delay the development of bone metastases as well as reduce the catastrophic complications of pain, hypercalcemia, fracture and nerve compression syndromes. It is already clear from clinical studies that the use of bisphosphonates, potent inhibitors of bone resorption, significantly reduces skeletal morbidity in advanced breast cancer (72-74). Bisphosphonates have also been shown to decrease the number of bone metastases in animal models (75,76), but it is unclear whether or not these tumors express PTHrP. However, since the safety of long term bisphosphonate use has not been determined and bone mineralization defects can occur with high doses of these drugs, it would be of benefit, as well as cost effective, to identify which patients are at risk to develop bone metastases and treat only those rather than treat all women with breast cancer. The clinical evidence thus far supports PTHrP as a marker to identify such women, but better animal models are needed to

clarify this role.

Knowledge of PTHrP status may also have significant therapeutic implications in treating breast cancer-associated hypercalcemia. Although hypercalcemia in breast cancer is often associated with bone metastases, it is clear that humoral mechanisms may contribute in as much as 60% of the cases. Traditionally, treatment has been directed toward inhibiting bone resorption and this is often effective. However, it has now become evident that bisphosphonate therapy is less effective in patients with higher plasma concentrations of PTHrP and without radiological evidence of bone metastases (77,78). Thus, inhibition of bone resorption is effective when the major mechanism for hypercalcemia is increased bone resorption. Since PTHrP causes hypercalcemia by both increasing osteoclastic bone resorption and increasing renal tubular reabsorption of calcium, drugs that inhibit bone resorption alone may not normalize the calcium concentration if the plasma PTHrP concentration is high enough to add a significant renal component to the hypercalcemia. Drugs directed against either the actions of PTHrP or the secretion of PTHrP may therefore be more beneficial in the bisphosphonate resistant situation. Unfortunately, no such drugs are available at the current time but the need for them is obvious. A potentially useful therapy may prove to be the use of monoclonal antibodies against PTHrP. Sato has recently described successful use of an anti-PTHrP-(1-34) monoclonal murine antibody in an animal model of humoral hypercalcemia that ameliorated hypercalcemia and prolonged survival time in severely ill animals (79).

In Vivo Models of Hypercalcemia and Osteolysis

These observations demonstrate the need for further study of the role of PTHrP in malignant and nonmalignant breast disease. The only research done to date on PTHrP expression in human breast cancer and its potential role in humoral hypercalcemia and the development of osteolytic bone metastases have involved the small clinical studies described above (24,25,39-41). Despite numerous animal models of human breast cancer (80) that have been described to date, human breast cancer cell lines have not been studied *in vivo* for PTHrP expression and its relationship to the development of osteolytic bone metastases and humoral hypercalcemia. Most animal models of breast cancer have been used to evaluate the effect of various factors (81-83) on breast cancer growth. Only one spontaneous rat mammary tumor (Walker 256 carcinosarcoma) has been shown to cause humoral hypercalcemia in rats (84), produce PTHrP (85) and cause osteolytic bone metastases (75). Given the accumulating evidence documenting a humoral mechanism for hypercalcemia in breast cancer, the established role of PTHrP in humoral hypercalcemia of malignancy, the presence of PTHrP in malignant as well as lactating breast tissue and the presence of PTHrP in established breast cancer metastases to bone, it is evident that established models of human breast cancer should be evaluated for PTHrP expression and its relationship to the skeleton. Using animal models will be beneficial in defining this aspect of the pathophysiology of breast cancer and this will in turn have important prognostic and therapeutic implications.

Historically, it has been difficult to produce bone metastases in animal models of malignancy. Tumors inoculated subcutaneously or intramuscularly do not metastasize in nude mice and tumors inoculated into the tail vein usually produce only lung metastases. Yoneda has developed an animal model of human breast cancer cell metastasis to bone (76,86) which is based on a model originally described by Arguello (87). In this model, MDA-MB-231 breast cancer cells injected into the left ventricle of nude mice reliably produce osteolytic lesions that are evident radiologically as well as histologically. This model has been used to show that the bisphosphonate, risedronate, decreased osteolytic lesions when given simultaneously with tumor cells and inhibited both an increase in new bone metastases and progression of each metastatic focus when given to animals with pre-existing osteolytic lesions (76).

PURPOSE OF PRESENT WORK

Breast cancer affects the skeleton through humoral and local osteolytic mechanisms to cause the devastating complications of hypercalcemia, pain, fracture and nerve compression syndromes. PTHrP is an important humoral mediator of hypercalcemia in cancer and may have physiologic roles in the lactating breast as well as in cell growth and differentiation. The role of PTHrP in the pathophysiology of breast cancer is significant for several reasons. 1) PTHrP mediates hypercalcemia through its systemic effects of increasing osteoclastic bone resorption as well as renal tubular calcium reabsorption in at least 50% of hypercalcemic breast cancer patients even in the presence of bone metastases. 2) Due to its potent bone resorbing capacity, PTHrP expression in the primary tumor may aid in establishment of the bone metastases that are so characteristic of patients with breast cancer. 3) Growth factors present in the bone microenvironment further enhance PTHrP expression in breast cancer cells present in bone and promote development of osteolytic lesions and tumor growth. Thus, PTHrP expression in the primary breast tumor may be a marker for the development of hypercalcemia and bone metastases.

The purpose of this study is to define the role of PTHrP in the pathophysiology of breast cancer using animal models of breast cancer-mediated humoral hypercalcemia and osteolytic bone metastases. Information gained from these studies will have important prognostic and therapeutic implications.

METHODS OF APPROACH

In order to define the role of PTHrP in the pathophysiology of breast cancer-associated hypercalcemia and skeletal complications in a systematic fashion, the following objectives were originally proposed.

1. **SPECIFIC AIM #1: To screen known breast cancer cell lines for PTHrP expression and secretion and to determine if PTHrP expression is related to estrogen receptor status.**
 - a. Known breast cancer cell lines (both estrogen receptor positive and negative) will be grown in culture along with positive and negative controls. Media conditioned for 24 hours will be screened for PTHrP immunoreactivity by immunoradiometric assay.
 - b. RNA will be isolated from above cell lines in the presence and absence of estrogen and PTHrP expression will be determined using Northern analysis.
2. **SPECIFIC AIM #2: Determine if known human breast cancer cell lines will cause humoral hypercalcemia and if this is PTHrP-mediated.**
 - a. Measure standard parameters of calcium homeostasis in nude mice bearing human breast tumors.
 - b. Determine that hypercalcemia observed in mice bearing PTHrP+ breast tumors is PTHrP-mediated. Two approaches will be used: i) to decrease PTHrP secretion by transfecting PTHrP + lines with PTHrP antisense ii) decrease PTHrP effects by administration of neutralizing antibody.
 1. Transfection of PTHrP antisense cDNA into breast cancer cell lines that secrete PTHrP and cause hypercalcemia in nude mice.
 2. Measurement of Ca^{++} in mice bearing hypercalcemic PTHrP+ breast cancer cell lines that have been transfected with PTHrP antisense cDNA.
 3. Measurement of Ca^{++} in mice bearing hypercalcemic PTHrP+ breast cancer cell lines that are treated with anti-PTHrP-(1-34) monoclonal antibody.

3. SPECIFIC AIM #3: To determine the role of PTHrP in the development of osteolytic metastases in breast cancer.

- a. Is PTHrP expression enhanced in the bone microenvironment relative to other metastatic sites? Using an animal model of breast cancer-mediated osteolysis, PTHrP expression will be compared in bone and non-bone sites using immunohistochemistry and in situ hybridization.
- b. Does expression of PTHrP in the primary tumor enhance the development and quantity of osteolytic bone metastases? Breast cancer cell line, MDA-231 will be transfected with the cDNA for human PTHrP or PTHrP-AS (antisense orientation as a control) and used in the osteolytic model.
 - 1. Production of stable MDA-231 clones expressing PTHrP or PTHrP-AS by calcium phosphate precipitation.
 - 2. Effect of MDA-231/PTHrP on development of osteolytic bone metastases will be assessed by inoculating these cells into the left ventricle of mice and determining if the quantity and size of the bone metastases differ from similarly inoculated control MDA-231/PTHrP-AS. Neutralizing antibodies will be given to attempt to block osteolysis in mice inoculated with MDA-231/PTHrP cells.

BODY

METHODS

Cell culture

The following cell lines were cultured in the respective media: RWGT2 (66), MDA-MB-231 (88), CHO-K1 and Hs578T in DMEM (Life Technologies, Grand Island, NY); BT549, ZR-75-1, T-47D and BT483 in RPMI (JRH Biosciences, Lenexa, KS) with 10 µg/ml bovine insulin (Biofluids, Rockville, MD); MDA-MB-435s in Leibovitz L-15 (Life Technologies); MCF-7 in IMEM (Biofluids) with 10 µg/ml bovine insulin. All media contained 10% FCS (Hyclone, Logan, UT), 0.1% penicillin/streptomycin and nonessential amino acids (Gibco). Cells were cultured in a 37°C atmosphere of 5% CO₂/air. MDA-MB-231 cells were provided by C. K. Osborne, M.D. (San Antonio, TX) and all other cell lines except RWGT2 were obtained from the American Type Culture Collection (Rockville, MD). To determine PTHrP concentration in conditioned media, cell lines were simultaneously plated onto 48-well plates at cell density of 10⁴/ml, grown to confluence and media (250 µl) were conditioned in the absence of serum for 48 hours. Cells were trypsinized and counted following collection of conditioned media. Media samples were stored at -70°C until assayed for PTHrP. For each cell line, PTHrP was measured in triplicate and corrected for cell number.

The effect of the bone-derived growth factor, TGFβ, on PTHrP secretion by breast cancer cell lines was investigated. Cells were plated in 48-well plates at 10⁴ cells/ml and grown to confluence. Media was replaced with serum-free DMEM with or without TGFβ (5 ng/ml). Conditioned media were collected after 48 hours. PTHrP concentrations were determined by IRMA and corrected for cell number.

PTHrP measurement

Immunoreactive PTHrP was measured in conditioned media and plasma using a 2 site IRMA kit available from Nichols Institutes. This assay utilizes 2 different polyclonal antibodies to PTHrP (1-40 and 60-72) and will detect only peptides that bridge these antibodies. All samples, conditioned media, plasma and bone marrow plasma were collected in protease inhibitors and stored at -70°C until assay, as PTHrP is very labile. The assay is performed on aliquots of fifty to two hundred microliters. PTHrP concentrations were determined from a standard curve using a computerized RIA program. The sensitivity of the assay is 0.3 pmol/L and specificity to human PTHrP-(1-86) is 100% and 0% with PTH. Intra-assay variation is between 2.9-9.5% and inter-assay variation between 5.3-5.6%.

Whole blood ionized calcium measurement

Whole blood ionized calcium concentrations (Ca²⁺) were measured using a Ciba Corning 634 ISE Ca⁺⁺/pH analyzer, which uses a sample volume of 35 µl. The calcium values are adjusted using the internal algorithm of the instrument pH of 7.4. Samples were run in duplicate and the mean value recorded. The analyzer were calibrated before and after running each group of samples using Ciba Corning 634 Slope Standard (Ca 2.50/pH 6.84). Many published studies have utilized this method (66,87,95).

Scoring of bone metastases

The number of osteolytic bone metastases was determined on radiographs as described by Nakai (86). At the end of the experiments, animals were anesthetized deeply with pentobarbital, laid down in a prone position against the films (22 x 27 cm X-O mart AR, Kodak) and exposed with an x-ray at 35 KVP for 6 seconds using a Faxitron Radiographic Inspection Unit (Model 8050-020, Field Emission Corporation, Inc.). Films were developed using a RP X-O Mart processor (Model M6B, Kodak). All of the radiographs of the bones in nude mice were evaluated extensively and carefully by 3 different individuals in a blinded fashion. From the radiographs, osteolytic metastatic foci as small as 1 mm in diameter, which are recognized as

demarcated radiolucent lesions in the bones, were enumerated.

Bone histology and histomorphometry. Forelimb and hindlimb long bones were removed from mice at sacrifice, fixed in 10% buffered formalin, decalcified in 14% EDTA and embedded in paraffin wax. Sections were cut using a standard microtome, placed on poly-L-lysine-coated glass slides and stained with hematoxylin, eosin, orange G and phloxine.

The following variables were measured in midsections of tibiae and femora, without knowledge of treatment groups, to assess tumor involvement: total bone area, total tumor area, and osteoclast number expressed per mm of tumor/bone interface. Histomorphometric analysis was performed on an OsteoMeasure System (Osteometrics, Atlanta, GA) using an IBM compatible computer.

In vivo experiments

A. Technical aspects of bone metastasis studies:

MDA-MB-231 breast cancer cells injected into the left ventricle of nude mice reliably produce osteolytic lesions that are evident radiologically as well as histologically. In this model, nude mice are inoculated into the left cardiac ventricle with a tumor cell suspension. In 3 weeks, osteolytic lesions are evident on radiographs. Histologic sections of these lesions reveal tumor adjacent to osteoclasts resorbing bone.

Tumor cells are grown to confluence, trypsinized, washed twice with PBS and resuspended with PBS to a final concentration of 10^5 cells/100 μ l immediately prior to inoculation. Animals are deeply anesthetized with pentobarbital (0.05 mg/g) and positioned ventral side up. The left cardiac ventricle is punctured through a percutaneous approach using a 27 gauge needle attached to a 1 ml syringe containing suspended tumor cells. Visualization of bright red blood entering the hub of the needle in a pulsatile fashion indicates correct position in the left cardiac ventricle. Tumor cells or PBS (for controls) are then inoculated slowly over 1 minute.

Three weeks following tumor inoculation, whole-body radiographs were obtained and compared to baseline radiographs obtained prior to tumor inoculation to follow the progression of osteolytic lesions. Ca^{++} and body weight were measured weekly for two weeks post tumor inoculation and then every three days thereafter for the remainder of the experiment. Animals were sacrificed when paraplegic or severely cachectic. At the time of sacrifice, blood was collected for Ca^{++} as well as PTHrP measurement and all bones and soft tissues were harvested in formalin for histologic analysis. Femurs with radiologic evidence of metastases from mice inoculated via the left cardiac ventricle with MDA-MB-231 cells or femurs from control mice were harvested by flushing marrow contents with 500 μ l of serum-free DMEM media into iced tubes containing EDTA and aprotinin for measurement of PTHrP in bone marrow plasma. Autopsy was performed on all mice and those with tumor adjacent to the heart were excluded from analysis as this indicates that part of the initial tumor inoculum did not properly enter the left cardiac ventricle.

B. Experimental design:

1. Experiment 1: PTHrP production in bone in vivo. Mice were inoculated into the left cardiac ventricle on day 0 with either MDA-MB-231 cell suspension or PBS after baseline radiographs were obtained, and Ca^{2+} and plasma PTHrP concentrations were measured (n=5/group). Radiographs were taken at day 21 and at sacrifice to follow the progression of osteolytic lesions. Ca^{2+} and body weight were measured on days 7, 14, 21, 24, and at sacrifice, day 26. At sacrifice, blood was collected for Ca^{2+} and PTHrP measurement, bone marrow plasma was collected for PTHrP measurement and all bones and soft tissues were harvested in formalin for histologic analysis. Autopsies were performed on all mice, and those with tumor adjacent to the heart were excluded from analysis as this indicated that part or all of the initial tumor inoculum had not entered the left cardiac ventricle.

2. Experiment 2: Effects of PTHrP antibody on MDA-MB-231-induced bone metastases.

a. On the development of new bone metastasis: Mice were divided into four treatment groups (n=7/group) and inoculated with MDA-MB-231 cells into the left cardiac ventricle on day 0. Treatment, administered at a dose of 75 µg subcutaneously twice per week starting 7 days prior to tumor inoculation and continued throughout the experiment, consisted of: 1) a murine monoclonal antibody directed against human PTHrP-(1-34) (PTHrP Ab; Mitsubishi, Japan); 2) control IgG (IgG; Sigma); 3) nothing; or 4) PTHrP Ab was given to a fourth group just prior to tumor inoculation and administered at the same dose and schedule for the remainder of the experiment. Radiography, Ca^{2+} and PTHrP measurement, bones and soft tissue harvest and autopsy were performed as in the previous experiment. Results from the 2 antibody groups were pooled in the final analysis as all parameters measured were similar regardless of whether the antibody treatment was initiated 7 days prior to or on the same day as tumor inoculation.

b. On the progression of established bone metastases: Eight female athymic nude mice were inoculated into the left cardiac ventricle on day 0 with nontransfected MDA-MB-231 cells. Osteolytic lesions were evident on radiographs at 2 weeks post inoculation and mice were divided into antibody treatment and control (PBS). Anti-PTHrP-(1-34) antibodies were administered subcutaneously twice per week beginning at this time in doses similar to those used in experiment 2a (70µg IgG).

Statistics

Unless otherwise indicated, values are reported as the mean \pm standard error of the mean. Statistical significance was determined using analysis of variance followed by Tukey's post test with Graphpad Prism software (San Diego, CA) on an IBM compatible computer.

RESULTS

In vitro experiments

Production of PTHrP by human breast cancer cell lines in vitro: Of the 8 breast cancer cell lines tested for PTHrP secretion *in vitro*, 4 produced low, but significant, amounts of PTHrP (Table 1). The PTHrP concentration in media conditioned by MDA-MB-231 cells was 5.4 ± 1.0 pM/ 10^6 cells/48 hours. This was significantly less than media conditioned by a squamous carcinoma of the lung, RWGT2, established from a patient with humoral hypercalcemia (66) (21.6 ± 2.3 pM/ 10^6 cells/48 hr.) but more than secreted by Chinese hamster ovarian (CHO) cells (undetectable).

TABLE 1

| CELL LINE | [PTHrP] (pM/ 10^6 cells/48 hours) |
|----------------------------|-------------------------------------|
| Human Breast Cancer | |
| MDA-MB-231 | $5.4 \pm 1.0^{**}$ |
| Hs578T | $4.6 \pm 0.5^*$ |
| BT549 | $4.4 \pm 1.2^*$ |
| MDA-MB-435s | 2.9 ± 1.2 |
| ZR-75-1 | N.D. |
| BT483 | N.D. |
| MCF-7 | N.D. |
| T-47D | N.D. |
| Human Lung Cancer | |
| RWGT2 | $21.6 \pm 2.3^{***}$ |
| Other | |
| CHOK1 | N.D. |

TABLE 1: PTHrP concentrations in conditioned media from cell lines. Collection of conditioned media is described in detail in the methods section. Results are expressed as the mean \pm the standard error of the mean. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with CHOK1. N.D. = not detectable. N = 3 wells per cell line. PTHrP concentrations in conditioned media from each cell line were corrected for cell number.

Figure 1 illustrates PTHrP secretion by 9 different breast cancer cell lines in response to stimulation by TGF β (5 ng/ml for 48 hours). All estrogen receptor positive cell lines (ER+), MDA-436, MDA-361, ZR-75-1, T-47D and BT-483, had undetectable concentrations of PTHrP in the conditioned media while all ER- lines, MDA-MB-231, BT-549, MDA-435s, Hs578T, and MDA-436 had low, but detectable PTHrP concentrations in the conditioned media. Of the 5 ER- cell lines that had measurable PTHrP, 4 of these cell lines increased production of PTHrP in response to TGF β . TGF β had no effect on PTHrP secretion by ER+ cell lines.

This work represents part of specific aim 1a that extends beyond studies reported in the last progress report. Estrogen regulation of PTHrP mRNA expression, as proposed in specific

aim 1b, is currently underway.

In vivo experiments

Experiment 1

MDA-MB-231 production of PTHrP in vivo: As part of specific aim 3a, PTHrP production by MDA-MB-231 cells was investigated *in vivo*. Mice inoculated with 10^5 MDA-MB-231 cells into the left cardiac ventricle developed radiographic evidence of osteolytic lesions over a period of 3 weeks. Mice were sacrificed at 4 weeks post tumor inoculation. Ca^{2+} and plasma PTHrP concentrations at sacrifice were not significantly different from respective values prior to tumor inoculation (1.28 ± 0.05 mM vs. 1.29 ± 0.03 mM for Ca^{2+} ; 1.04 ± 0.06 pM vs. 1.05 ± 0.09 pM for PTHrP). In contrast, PTHrP concentrations in bone marrow plasma harvested from femurs affected with osteolytic lesions were significantly higher than corresponding plasma PTHrP concentrations (2.46 ± 0.34 pM vs. 1.05 ± 0.09 pM, $p < 0.001$; Figure 2). Bone marrow plasma PTHrP concentrations from femurs of nontumor-bearing mice were below the detection limit of the assay. Thus, PTHrP produced by normal bone marrow cells was not of sufficient quantity to be detected by this method.

Experiment 2

PTHrP antibody experiments in vivo: As part of specific aim 3b, to determine if expression of PTHrP in the primary breast tumor enhances the development and quantity of osteolytic bone metastases, mice inoculated with MDA-MB-231 cells were studied in the mouse metastasis model with or without treatment with PTHrP antibodies. Since experiment 1 suggested that local PTHrP production by cancer cells in bone may be important in breast cancer-mediated osteolysis, the next experiment was designed to determine the role of PTHrP in the development of MDA-MB-231-mediated osteolysis. Nude mice were treated with a murine monoclonal antibody directed against PTHrP-(1-34) prior to intracardiac inoculation of tumor cells and compared with similarly inoculated animals treated with control IgG or nothing. PTHrP Ab and control IgG were administered at a dose of 75 μ g twice per week throughout the experiment. Mice were sacrificed 26 days after tumor inoculation. One mouse each from the no treatment group and the PTHrP Ab group were excluded from analysis as tumor was adjacent to the heart at autopsy.

Figure 3 illustrates representative radiographs taken 26 days after tumor inoculation. Obvious osteolytic lesions were present in mice that received no treatment or control IgG, while very few metastatic lesions were present in mice treated with the PTHrP Ab. The total area of radiographic osteolytic lesions from all long bones was quantified by a computerized image analysis system (Figure 4). Lesion area was significantly less in mice treated with the PTHrP Ab compared with mice given no treatment or control IgG ($p < 0.001$). Values in the latter two groups were not statistically different. Representative histologic sections through the proximal tibial metaphysis are illustrated in Figure 5. Tumor filled the bone marrow space and destroyed both trabecular and cortical bone in mice that received no treatment or control IgG. In contrast, most of the PTHrP Ab-treated mice had intact cortical and trabecular bone and many bones had no evidence of tumor involvement. When tumor was present in the bone marrow space in PTHrP Ab-treated mice, it was often present as small, discrete foci within the marrow cavity and associated with little or no bone destruction.

Histomorphometric analysis of the hindlimbs from mice in all treatment groups confirmed radiographic quantitation of the osteolytic lesion area (Figure 6). Tumor area (Figure 6A) and osteoclast number per mm of tumor/bone interface (Figure 6B) were significantly less in mice treated with PTHrP Ab compared with the mice that received no treatment or control IgG. Residual bone area was significantly higher in the PTHrP Ab-treated mice compared with the controls (Figure 6C).

Ca^{2+} concentrations remained normal in all groups for the duration of the experiment (1.26 ± 0.03 mM (PTHrP Ab), 1.28 ± 0.02 mM (no treatment), 1.28 ± 0.02 mM (IgG); $p = \text{ns}$; Figure 7A). Body weight significantly declined in mice that received no treatment or control IgG

compared with those treated with PTHrP Ab (Figure 7B). These weight differences reached statistical significance on day 26 (21.6 ± 0.6 g (PTHrP Ab) vs. 18.8 ± 0.8 g (no treatment) and 17.7 ± 0.8 g (IgG); $p < 0.05$). Additionally, no differences were evident between PTHrP Ab-treated and control mice regarding tumor cell metastases to sites other than bone. Gross and histologic examination of soft tissues revealed adrenal gland metastasis in one mouse each from the no treatment group and the PTHrP Ab group.

In a separate experiment to determine if treatment with PTHrP antibodies could delay the progression of established MDA-MB-231 bone metastasis, female athymic nude mice with radiographic osteolytic lesions 2 weeks post tumor inoculation were treated with either PTHrP-Ab or control (PBS) subcutaneously twice per week. Figure 8 demonstrates that total radiographic lesion area was less in those animals treated with the PTHrP-Ab compared with controls ($p < 0.001$).

CONCLUSIONS

These findings demonstrate that nude mice, following inoculation with MDA-MB-231 cells into the left ventricle, develop metastatic bone disease with the same characteristic features as those seen in breast cancer patients. Tumor-bearing mice not only have osteolysis without increased plasma calcium or PTHrP concentrations but also have enhanced production of PTHrP by tumor cells in the bone microenvironment. In our experiments, this was evidenced by the increased PTHrP concentrations in the bone marrow plasma of affected femurs compared with blood plasma. Thus, the concentrations of PTHrP secreted *in vivo* by MDA-MB-231 cells are presumably sufficient to mediate local osteolysis, but not enough to have the systemic effects that characterize humoral hypercalcemia. Treatment with PTHrP monoclonal antibodies in this model of breast cancer-mediated osteolysis resulted in marked inhibition of the development of new osteolytic lesions and decreased osteoclast number per millimeter of tumor/bone interface, indicating that PTHrP is the critical mediator of bone destruction in this situation.

Several clinical studies in breast cancer patients indicate that plasma PTHrP concentrations are increased in approximately 50% of those with hypercalcemia (89-91). These data, along with the fact that one of the 3 tumors from which PTHrP was originally purified (10-12) was a breast carcinoma associated with humoral hypercalcemia, show that PTHrP may mediate hypercalcemia in some patients with breast cancer. In addition to this established role of PTHrP in malignancy-associated hypercalcemia, the findings presented here implicate PTHrP in the causation of breast cancer-mediated osteolysis even in the absence of hypercalcemia or increased plasma PTHrP concentrations.

One issue which arises is whether local PTHrP production by breast cancer cells in bone is a common phenomenon, and how many patients with metastatic breast cancer to bone would benefit from neutralization or inhibition of PTHrP. This issue remains to be resolved. However, our survey of PTHrP production in breast cancer cell lines demonstrate that 5 out of 9 cell lines tested secrete significant amounts of PTHrP. These data support the clinical studies which demonstrate PTHrP expression in primary breast cancer by immunohistochemical methods to be approximately 50-60% (24). The clinical observations that primary breast tumors which express PTHrP are associated with the development of bone metastases (39,40) and that PTHrP expression by breast cancer cells in bone is greater than that of tumor cells which have metastasized to non-bone sites (25) or primary breast tumors (24) are consistent with the *in vivo* data presented here.

These data and those of others show that PTHrP is frequently produced by human breast cancer cells *in vitro* and *in vivo*, and that neutralization of PTHrP may inhibit development or progression of osteolytic metastases, but they do not exclude the involvement of other mediators. Cytokines, such as tumor necrosis factor, interleukin-6 or interleukin-1 produced locally by tumor cells or normal host cells, in response to the tumor, have the capacity to stimulate osteoclastic bone resorption (92). Such mediators have also been shown to

modulate the end-organ effects of PTHrP (66,93) as well as to increase its secretion from tumor cells (94). Thus, other locally-produced osteolytic factors may contribute to breast cancer-induced bone destruction as well.

Immunohistochemical data from patients with metastatic breast cancer suggest that PTHrP production by tumor cells is enhanced in the bone microenvironment (25). Our data are in agreement with this concept. The capacity of breast cancer cells to express PTHrP may give them a growth advantage after they have metastasized to bone, due to the ability of PTHrP to increase osteoclastic bone resorption (17). Growth factors, such as transforming growth factor beta (TGF β) and insulin-like growth factors I and II are present within bone matrix (70) and released into the bone microenvironment as a result of osteoclastic bone resorption (71). Such bone-derived growth factors are likely to be in high concentration and in close proximity to tumor cells in bone. TGF β enhances PTHrP expression by breast (95) and other cancers (96) and insulin-like growth factors may modulate breast cancer growth (97). Thus, once cancer cells in bone stimulate osteoclastic bone resorption, they may initiate a vicious cycle in which growth factors released from matrix enhance tumor cell growth and PTHrP production. This leads to more aggressive local bone resorption and a more favorable environment for further tumor growth and subsequent bone destruction. Since normal bone is actively remodeling, and growth factors are being released locally, PTHrP expression may be stimulated once the breast cancer cells lodge in the bone marrow stroma.

We found a marked decrease in tumor area in mice treated with PTHrP Ab, which is consistent with the notion that tumor growth is positively correlated with rates of bone resorption. In further support that neutralization of PTHrP leads to a decrease in tumor burden, tumor-bearing mice treated with PTHrP Ab maintained normal weight whereas the controls lost a significant amount of body weight. A possible explanation is that the neutralizing antibodies to PTHrP had a direct effect on tumor growth, but this is unlikely since tumors growing in non-bone sites were not affected by PTHrP Ab, and PTHrP Ab did not affect tumor cell proliferation *in vitro*. The most likely explanation is that the antibodies neutralized the biological activity of PTHrP, thereby preventing the increase in osteoclastic bone resorption and the release of growth factors from bone which may enhance growth of the tumor cells locally. This mechanism is supported by studies demonstrating that tumor burden in bone was decreased in mice treated with bisphosphonates, selective inhibitors of osteoclastic bone resorption (76).

The observation that normal body weight was maintained in tumor-bearing mice suggest that limiting development of bone metastases with PTHrP Ab did not lead to enhanced tumor growth in other organ sites. However, the overall effect of this treatment on tumor metastasis to organ sites other than bone remains to be explored. What is clear is that neutralizing antibodies prevented destructive bone lesions and also reduced tumor mass in bone.

These data have important implications for the management of patients with breast cancer-mediated osteolysis. First, treatment directed against PTHrP, such as the antibodies used in these experiments, may prevent the development of new bone metastases and delay the progression of established metastases. Second, PTHrP expression by the primary breast tumor may be a marker for increased capacity to form bone metastases. Third, treatment with inhibitors of PTHrP or inhibitors of osteoclastic bone resorption such as bisphosphonates may be effective adjuvant therapies not only for the prevention and treatment of bone metastases, but also for reducing tumor burden by making bone a less favorable site for continued tumor growth.

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FIGURE 1

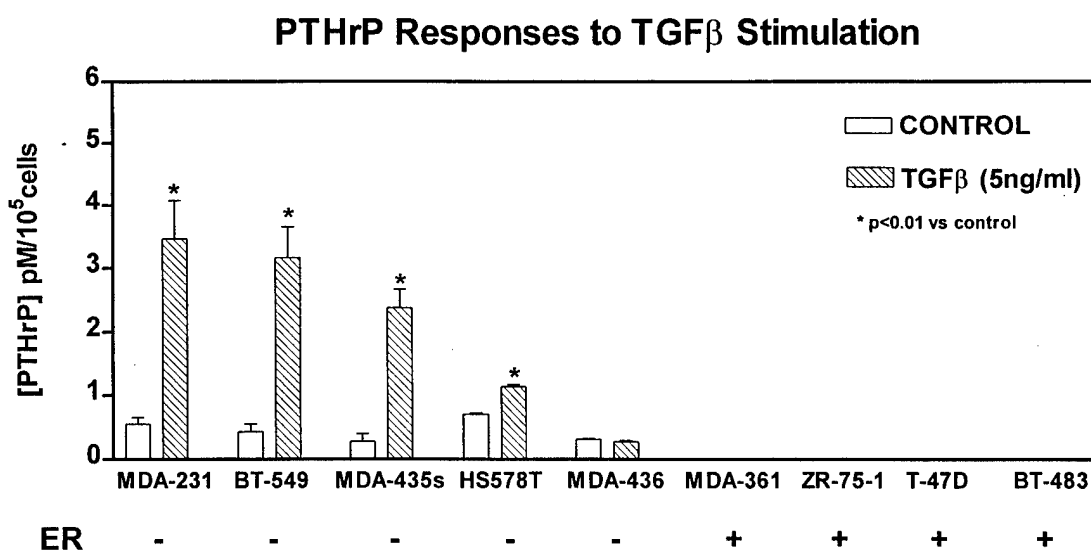


FIGURE 1: PTHrP secretion by 9 different breast cancer cell lines in response to stimulation by TGF β (5 ng/ml for 48 hours). Cells were plated in 48-well plates at 10⁴ cells/ml and grown to confluence. Media was replaced with serum-free DMEM with or without TGF β (5 ng/ml). Conditioned media were collected after 48 hours. PTHrP concentrations were determined by IRMA and corrected for cell number. All estrogen receptor positive cell lines (ER+), MDA-436, MDA-361, ZR-75-1, T-47D and BT-483, had undetectable concentrations of PTHrP in the conditioned media while all ER- lines, MDA-MB-231, BT-549, MDA-435s, Hs578T, and MDA-436 had low, but detectable PTHrP concentrations in the conditioned media. Of the 5 ER- cell lines that had measurable PTHrP, 4 of these cell lines increased production of PTHrP in response to TGF β . TGF β had no effect on PTHrP secretion by ER+ cell lines.

FIGURE 2

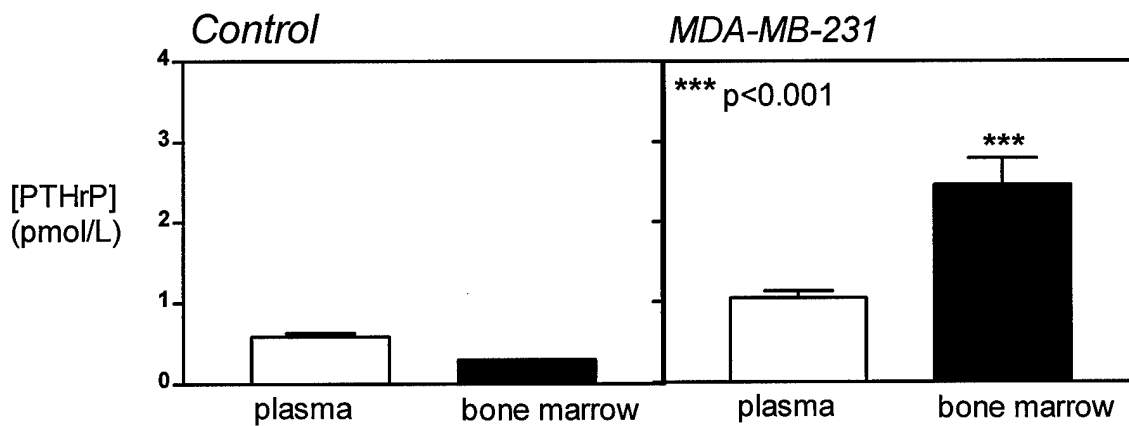


FIGURE 2: Simultaneous PTHrP concentrations in plasma and bone marrow plasma from nontumor-bearing mice (control; left panel) or mice with bone metastases due to MDA-MB-231 tumor (right panel). Bone marrow plasma (indicated as bone marrow in figure) was obtained at the time of sacrifice from femurs with radiologic evidence of bone destruction in mice with MDA-MB-231 tumors or from nontumor-bearing femurs in control mice. Plasma samples were obtained from whole blood collected from respective mice at the same time. N=5 mice/group. Results are expressed as the mean \pm the standard error of the mean. ***p<0.001 compared with concomitant plasma sample in mice with MDA-MB-231 tumors.

FIGURE 3

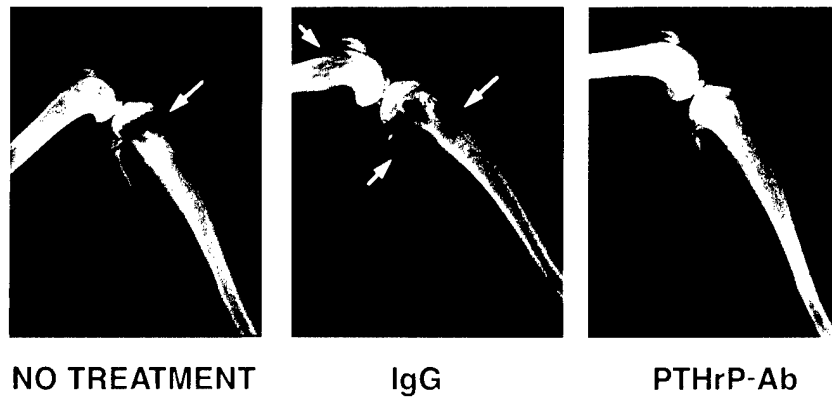


FIGURE 3: Representative radiographs of osteolytic bone lesions in hindlimbs from mice inoculated via the left cardiac ventricle from respective treatment groups. Radiographs were taken 26 days after tumor inoculation with MDA-MB-231 cells. Arrows indicate osteolytic metastases in distal femur, proximal tibia and fibula.

FIGURE 4

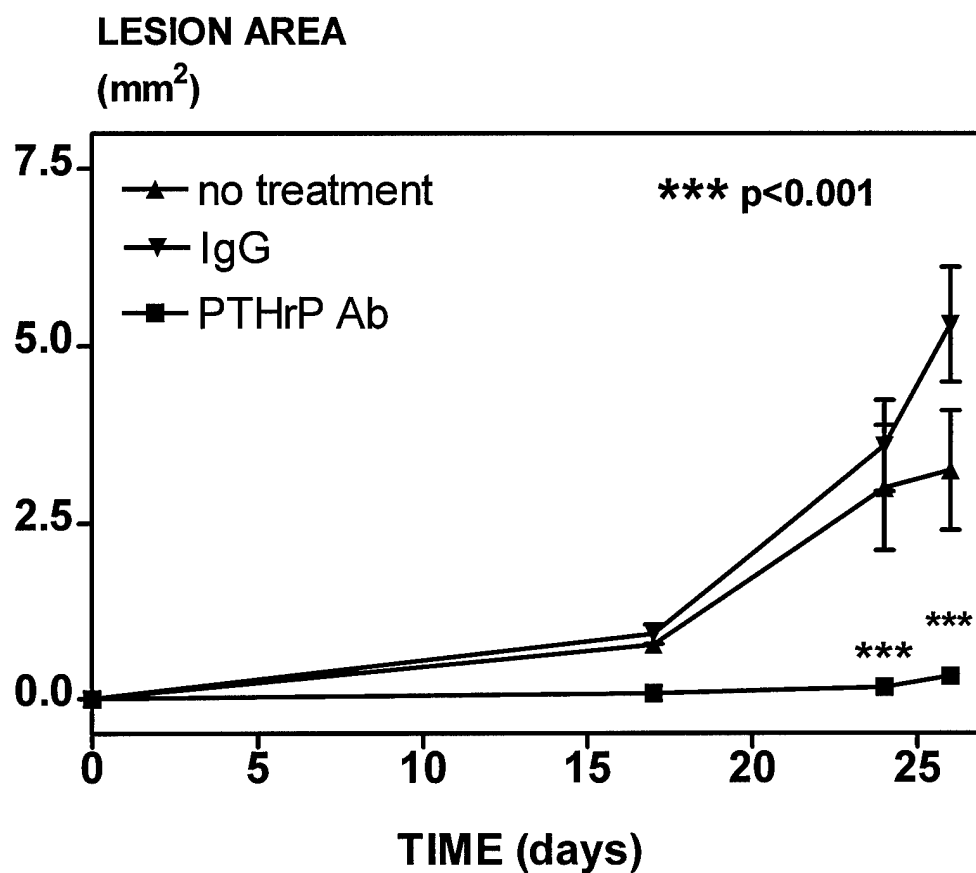


FIGURE 4: Osteolytic lesion area on radiographs as assessed by computerized image analysis. MDA-MB-231 cells were inoculated on day 0, and representative treatment (IgG or PTHrP Ab) started 7 days prior to tumor inoculation and continued twice per week throughout the experiment. Lesion area was measured from long bones of fore- and hindlimbs. N=7 mice/group. All values represent the mean \pm SEM. Standard error bars are not visualized at some of the time points as they fall within the size of the symbol.

FIGURE 5

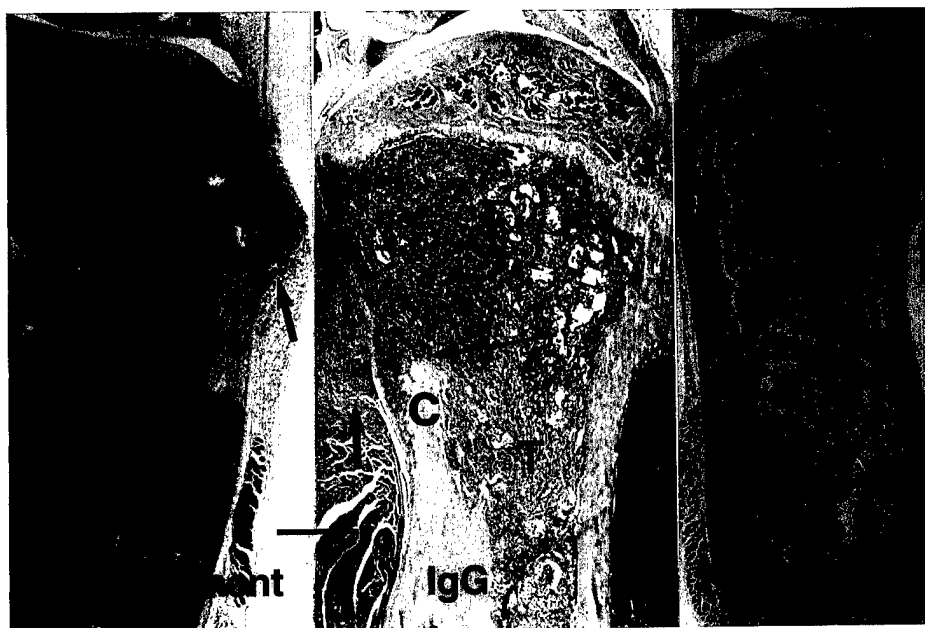


FIGURE 5: Effect of PTHrP antibody on MDA-MB-231 cell metastasis to murine tibiae. MDA-MB-231 breast cancer cells were injected into the left ventricle of nude mice which were sacrificed 26 days later. Mice were also given 75 μ g of PTHrP antibody or IgG twice weekly or else received no concomitant treatment. The left hand and middle panels illustrate representative sections of the tibiae from mice given no treatment or IgG. Most of the cancellous bone in the primary and secondary spongiosae has been replaced by metastatic tumor cells (T) which almost completely fill the bone marrow cavity. The cortical bone (C) has also been destroyed by osteoclasts at the proximal ends of the bones (large arrows) in response to the metastatic cancer cells. Tumor cells have spread through the cortical bone into the surrounding soft tissues (small arrows). In contrast, the right hand panel illustrates the tibia from a representative mouse injected with tumor cells and given PTHrP antibody. A relatively small deposit of metastatic tumor (T) is present within the bone marrow cavity distal to the secondary spongiosae. The bone trabeculae at the primary and secondary spongiosae are preserved (small arrows), appear normal and are surrounded by normal bone marrow hematopoietic tissue. The bar represents 555 μ m. Hematoxylin, eosin, phloxine and orange G staining.

FIGURE 6

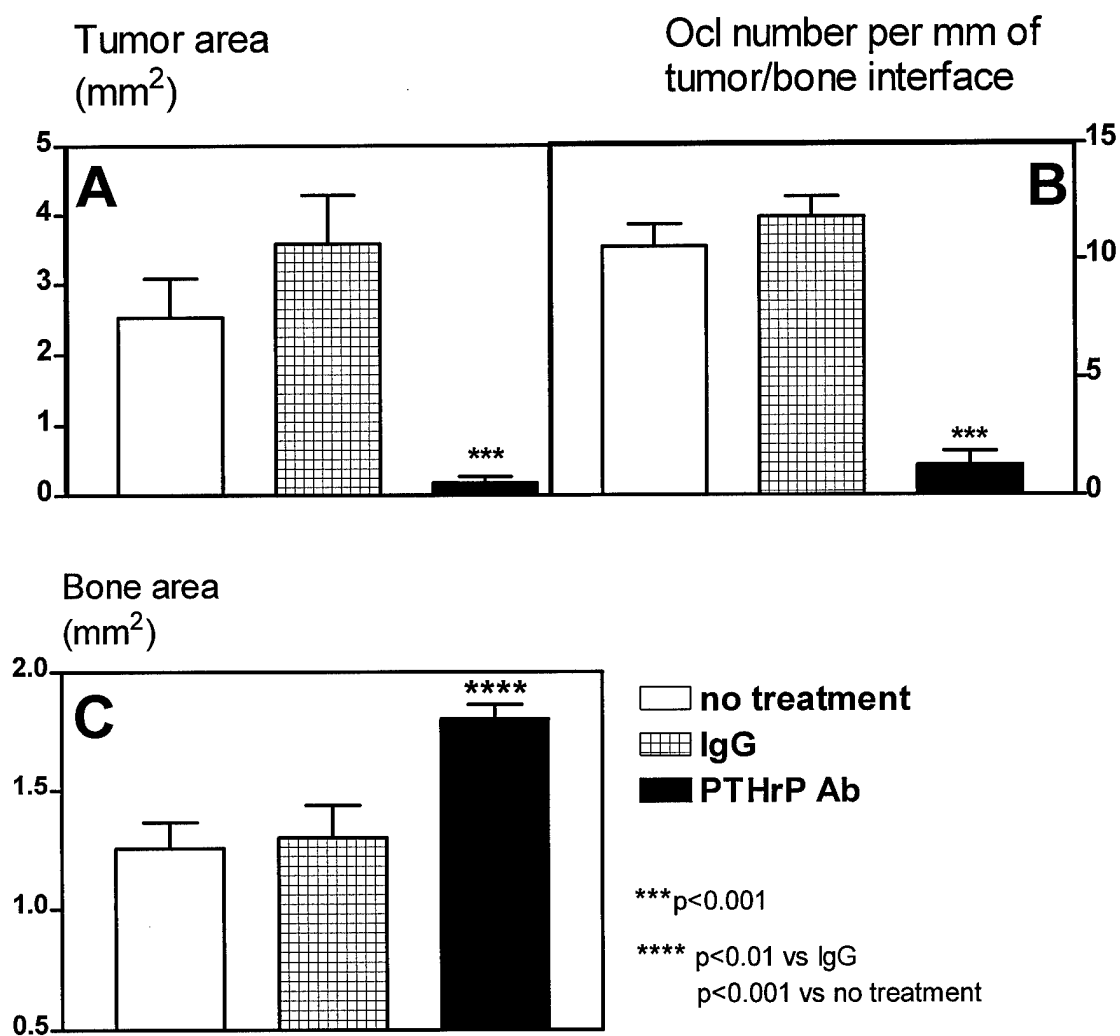


FIGURE 6: Histomorphometric analysis of hindlimbs from tumor-bearing mice with MDA-MB-231 metastatic bone lesions. Data represent measurements from tibiae and femurs of mice from figure 3 that were treated with either PTHrP Ab, IgG or nothing. A) Tumor area (mm²) from MDA-MB-231 metastatic bone lesions. B) Osteoclast number per mm of tumor adjacent to bone (tumor/bone interface). C) Total bone area (mm²) was measured in one low-power magnification field (4x) at the head of the tibia or femur, the site of most bone destruction. All values represent the mean \pm the standard error of the mean.

FIGURE 7

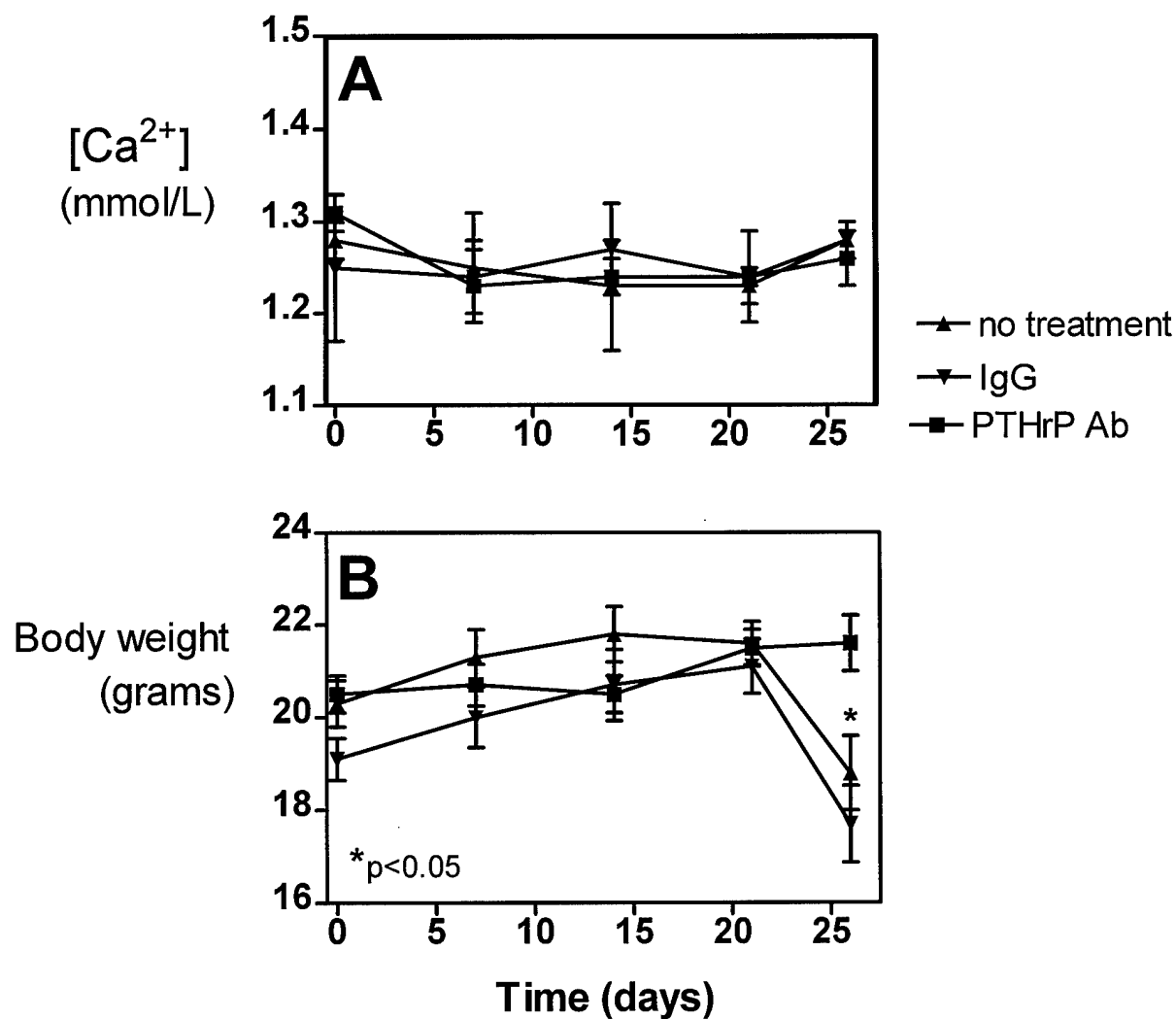
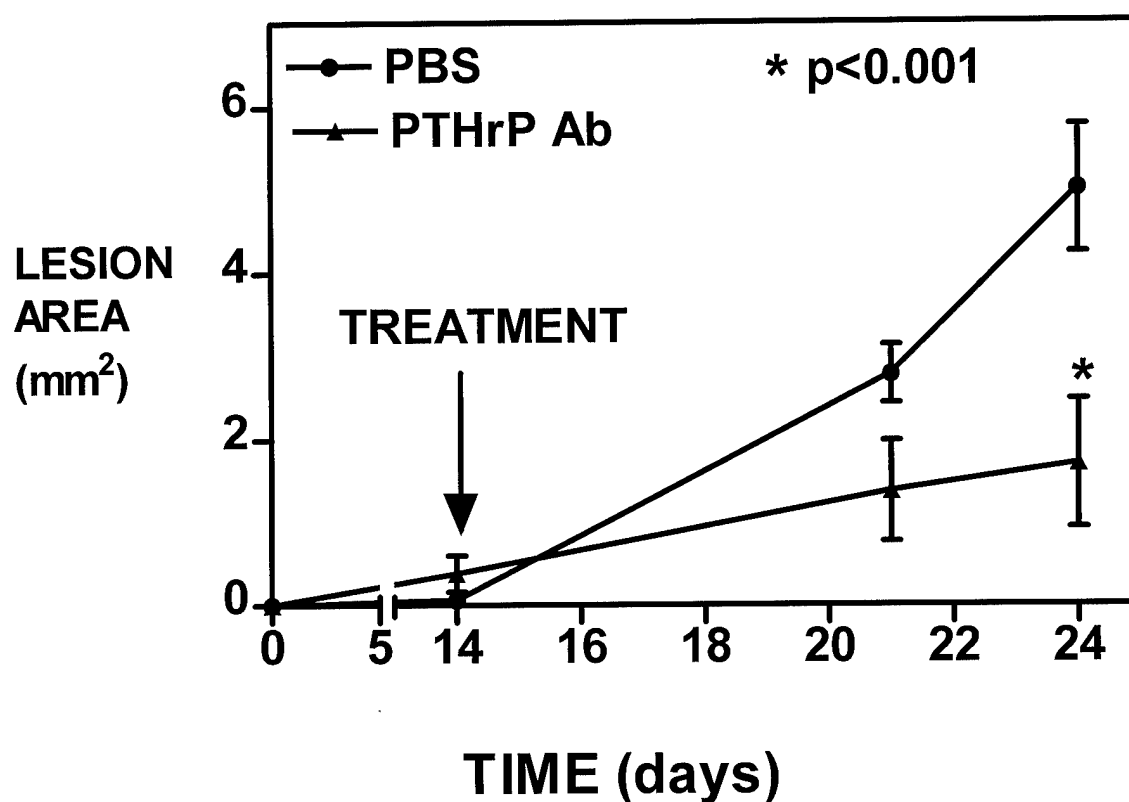


FIGURE 7: Whole blood ionized calcium concentrations [Ca²⁺] (A) and body weight (B) in tumor-bearing mice from figure 3 that received either PTHrP Ab, control IgG or no treatment. There were no significant differences between the groups with regard to [Ca²⁺] (A) or body weight (B). All values represent the mean \pm the standard error of the mean.

FIGURE 8

PTHrP Ab ON ESTABLISHED METASTASES



Area of osteolytic lesions in mice with established bone metastases. Eight female athymic nude mice were inoculated into the left cardiac ventricle on day 0 with nontransfected MDA-MB-231 cells. Osteolytic lesions were evident on radiographs at 2 weeks post inoculation and mice were divided into antibody treatment and control (PBS). Anti-PTHrP-(1-34) antibodies were administered subcutaneously twice per week beginning at this time in doses similar to the previous experiment (70µg IgG).